Original Article

**CD4⁺CD29⁺ T cells are blamed for the persistent inflammatory response in ulcerative colitis**

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**Abstract:** Ulcerative colitis (UC) is a chronic gastrointestinal disorder eliciting occurrence of colorectal cancer, the third most common human malignancy. The diagnosis of UC is based on clinical symptoms combined with typical findings on endoscopy, radiology, and ultimately pathology. We investigated the variation trend of CD4⁺CD29⁺ T cells together with MPO, VCAM-1 in different periods of rat UC model and UC patients. We also evaluated the relationship between CD4⁺CD29⁺ T cells and disease severity. UC model was induced by administering DNCB liquid and acetate solution. We found upregulated expression of CD4⁺CD29⁺ T cells in both peripheral blood and colon from rats, and a similar trend for MPO and VCAM-1 in colon (P < 0.05); the expression was especially enhanced in UC rats at two weeks after the model was established (P < 0.01). Such upregulation was also indicated in active and remission UC patients as compared to the healthy and enteritis groups (P < 0.05), with the highest expression level detected in the active UC patients (P < 0.01). Pearson correlation analysis showed a positive correlation of CD4⁺CD29⁺ T cells in rat and human peripheral blood with DAI score (r<sub>rat</sub> = 0.712, r<sub>human</sub> = 0.677, P < 0.01), and MPO in colon (r<sub>rat</sub> = 0.514, r<sub>human</sub> = 0.682, P < 0.05). These results suggest that CD4⁺CD29⁺ T cells may act as major effector cell subsets in persistent inflammatory responses for UC and that infiltration into colon inflammation may be induced by the combination of VCAM-1 and CD29.

**Keywords:** Ulcerative colitis, CD4⁺CD29⁺ T cells, VCAM-1, persistent inflammatory responses

**Introduction**

Ulcerative colitis (UC), whose etiology remains unclear, is a chronic relapsing and remitting inflammatory disorder of the gastrointestinal tract [1]. The regulatory T cells in intestinal tract known for their inability to balance the excessive inflammatory responses against intestinal flora and food antigens are considered an important immune mechanism of UC [2, 3]. The role of main effector cell subsets in excessive inflammatory responses for UC, however, remains to be elucidated.

CD4⁺T cells serve as a major effector cells and are frequently overexpressed in UC [4]. CD4⁺CD29⁺T cells belong to memory effector cells. CD29 is preferentially expressed on activated T cells that respond very well to recall antigens [5-7]. Activation of beta (1) integrins (CD29) has been reported to mediate proliferation and inhibit apoptosis of intestinal CD4⁺ positive lymphocytes, important factors to perpetuate inflammatory process [8]. Existing studies suggested that enhanced expression of adhesion molecules might lead to accumulation of CD4⁺CD29⁺ T cells in inflamed tissues [9]. The aforementioned data led to the hypothesis that CD4⁺CD29⁺ T cells might act as main effector cell subsets in persistent inflammatory responses for UC, which could be migrated to inflamed tissues through VCAM-1, a from of adhesion molecule and the ligand of
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As yet, there are sparse data on association between variation of CD4+CD29+ T cells and UC, highlighting the need for additional investigations to identify the role CD4+CD29+ T cells have on the serious chronic inflammatory disease.

Herein, we analyzed the change trend of CD4+CD29+ T cells, VCAM-1 and myeloperoxidase (a vital indicator of neutrophilic-granulocyte in active UC) at different phases for rat and human UC. In addition, we explored the relationship of CD4+CD29+ T cells to DAI score and MPO.

Methods and materials

Animals and experiment design

Experiments approved by Guangdong Medical Laboratory of Animal Center were performed using 2-month-old female SD rats (200-220 g). Thirty-six rats were maintained under standard conditions for two weeks of acclimatization. Among these, twenty-eight were randomly taken as cases and the remainders as normal controls. The UC model was established as detailed elsewhere [10], with small changes. In brief, all rats were administered 0.25 mL of 20 g/L DNCB acetone liquid (DNCB, Sigma, USA) solution on their bare necks once daily for 14d after depilation with Na₂S (100 g/L). At day 15, 0.25 mL of 20 g/L DNCB acetone liquid was applied to their bare abdomen after depilation with Na₂S. The bare abdomen that turned red and swollen the next day was considered immune sensitization. At day 16 and 17, 0.25 mL of 0.1% DNCB ethanol solution was infused through a nylon hose inserted into the colon of rat (8 cm in depth). At day 18, 2 mL 8% acetate solution was administered to their bare abdomen after depliation with Na₂S. The bare abdomen that turned red and swollen the next day was considered immune sensitization.

At day 16 and 17, 0.25 mL of 0.1% DNCB ethanol solution was infused through a nylon hose inserted into the colon of rat (8 cm in depth). At day 18, 2 mL 8% acetate solution was administered to their bare abdomen at the same site. Then, the colon was rinsed with 5 mL normal saline after 10s of retention. After that, all rats were housed under standard conditions and the general situations such as diet, hair, and excrement were observed. We obtained 24 survival rats that were randomized into 3 groups. They were killed at day 1, 2 weeks, 6 weeks, respectively, after the model was stable (labeled as 1 day, 2 weeks, 6 weeks). Heparinized peripheral blood was collected for CD4+CD29+ T cells by flow cytometry. Moreover, 7, 6, 5, 5 intestinal tissues were obtained from the active UC, remission, infective enteritis and healthy control group, respectively, then fixed by formalin and imbedded by paraffin immediately after excision for HE hemotoxylin and eosin staining and immunohistochemistry of MPO and VCAM-1. All patients provided informed consent.

Flow cytometric analysis

Peripheral mononuclear cells (PBMC) obtained from heparinized peripheral blood were prepared for flow cytometry analysis based on previously described methods [14]. Briefly, 1 x 10⁶ cells per sample were incubated (30 mins, on ice) with PE and APC-conjugated MoAb to antigen CD4 and CD29 (Biolegend, California, USA). PE and APC-labeled isotype-matched control Ig (Biolegend, California, USA) were also incubated. Double fluorescent analysis was conducted using a Facscan flow cytometer (Becton, Dickinson). Results were expressed as percentage of CD4+ T cells.

Immunofluorescence

To analyze the expression of CD4+CD29+ T cells in colon, intestinal tissues were frozen for immunofluorescence referring to the previous literature [15], with minor changes. The frozen sections were fixed in cold acetone for 10 min,
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and sealed in 10% of normal goat serum. Then the mixture was incubated in a cocktail containing non-conjugated MoAb to antigen CD4 (Rabbit IgG, Gene Tex) and CD29 (mouse IgG, BD) at 4°C for 12 h, soaked in a cocktail containing secondary Goat anti-rabbit-488 and Goat anti-mouse-594 (Shanghai research set) at room temperature without light for 1 h, and finally blotted and mounted with DAPI-contained anti-fade medium under a coverslip. Images were acquired by Image-Pro Plus microscope and processed with Emage-Pro Express software.

**Immunohistochemistry**

In order to explore the possible correlative factors for CD4⁺CD29⁺T cells, MPO and VCAM-1 in rat and human colon were measured by immunohistochemistry staining as previously detailed [16]. 5-µm paraffin-embedded sections were dehydrated. Antigen retrieval was completed in microwave oven and then incubated with mouse anti-rat and mouse anti-human MPO (1:100) and VCAM-1 (1:100) antibody at 4°C overnight, followed by treatment with biotinylated goat anti-mouse immunoglobulin (1:300 dilution) for 30 min, and staining with DAB. Immunostaining density was assessed by counting five randomly selected microscope fields and analyzed using Image Pro Plus version 6.0 (Maryland, USA) (results were presented as a ratio).

**Statistical analysis**

All data were analyzed using SPSS16.0 and expressed as means ± standard error (SE). The
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one-way analysis of variance was used for statistical analyses to compare the differences among multiple groups. Pearson’s method was used to analyze the correlations of DAI and MPO with CD4+CD29+ T cells in rat and human peripheral blood. Statistical significance was defined as P < 0.05.

Results

DAI, pathology performance and MPO status

DAI, pathology performance and MPO are important indicators of symptoms, histology and immune reactions to assess activity or severity of UC [11, 12, 17]. DAI was determined by scoring the extent of body weight loss, stool haemoccult positivity or gross bleeding, and stool consistency [11]. We observed higher DAI in UC model groups than in the normal group (P < 0.01), with the highest DAI detected in 2 weeks group and relatively lower in 6 weeks group (P < 0.05) (Figure 1D). A similar variation trend was shown in pathology performance. At 2 weeks, diffuse inflammatory cellular infiltration, erosion and ulcers were present in epithelial mucosae and submucosa. At 6 weeks, the number of inflammatory cells sharply decreased, with lymphocytes infiltration in mesenchyme and fiber hyperplasia (Figure 1A). MPO was used as a sensitive marker to assess disease activity [17], and the 2 weeks group showed the highest expression levels among all groups (P < 0.05). The 6 weeks group again showed significantly lower levels than the 2 weeks group (P < 0.05). Immunohistochemistry was performed under the same experimental conditions in order to evaluate the expression status. We observed significantly upregulated MPO in enteritis and UC patients compared to healthy controls, with more obvious upregulation seen in the active UC patients in comparison to the enteritis and remission group (P < 0.05) (Figure 1B, 1C).

Upregulated expression of CD4+CD29+ T cells in peripheral blood

To test the hypothesis that CD4+CD29+ T cells may act as main effector cell subsets in persistent inflammatory responses for UC, we analyzed CD4+CD29+ T cells in peripheral blood of UC rats and patients. As shown in Figure 2A, we did not find a significant rise at day 1 after model was stable (P = 0.401), but we did observe a notable increase at 2 weeks (P < 0.01), and a sharp decrease in the next four weeks (P < 0.01).

In human peripheral blood, we observed a higher percentage of CD4+CD29+ T cells as compared with the healthy and enteritis group (P < 0.05). We also noted a significantly larger number in the active UC group when compared to...
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**Figure 3.**

A. Immunofluorescence of CD4⁺CD29⁺ T cells expression in rat and human colon (the yellow section that arrows point to was expression of CD4⁺CD29⁺ T cells, (a-d) × 400). (a, e) no obvious expression. (b, f) a little and weak expression. (c, g) strong fluorescence intensity. (d, h) a little and weak expression. B. Immunohistochemistry
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of VCAM-1 expression in rat colon, (a-d) × 200. (a) no obvious expression (b) a little expression, mainly in mucosa (c) much more expression than 1 day group, mainly in mucosa and submucosa (d) a little expression, mainly in mucosa. C. IOD of VCAM-1 in rat and human colon, analyzed using Image Pro Plus version 6.0 (Maryland, USA) (results were presented as a ratio). *P < 0.05 as compared with the 1 day and enteritis group, **P < 0.05 as compared with the 2 weeks and active UC group.

Enhanced expression of CD4⁺CD29⁺T cells and VCAM-1 in colon

As UC was considered an inflammatory response prone to initiate colon cancer [18], thus it is substantially important to analyze the expression of CD4⁺CD29⁺T cells in colon. Immunofluorescence analysis was performed to detect the expression status. We also investigated the expression of VCAM-1, an adhesion molecule and the ligand of CD29, using Image Pro Plus version 6.0. No obvious expression of CD4⁺CD29⁺T cells or VCAM-1 was found in reference groups. Interestingly, different degrees of expression were detected in UC groups. The CD4⁺CD29⁺T cells were sparsely distributed and predominantly expressed in cell membrane, while VCAM-1 was mainly detected in mucosa and submucosa. We found significantly upregulated VCAM-1 in 2 weeks rat group and the active UC group (P < 0.05). In addition, CD4⁺CD29⁺T cells and VCAM-1 showed the widest range and the strongest

Correlation analysis

Figure 4. A. Correlation between DAI and CD4⁺CD29⁺T cells in peripheral blood of rat and human. B. Correlation between MPO in colon and CD4⁺CD29⁺T cells in peripheral blood of rat and human. Pearson’s t test was used to analyze the correlation; P values are indicated.

the remission group (P < 0.01). Nevertheless, no statistically significant difference was indicated between the health group and the enteritis group (P = 0.630, Figure 2A). CD4⁺CD29⁺T cells in peripheral blood showed consistency with UC patients in DAI, pathology performance and MPO, suggesting CD4⁺CD29⁺T cells may be closely related to UC activity and severity (Figure 4).

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intensity in 2 weeks rat group and the active UC group (Figure 3A, 3B).

cells was found in patients with atopic diseases testing that a lower percentage of CD4 autoimmune diseases such as multiple sclerosis demonstrated is in line with previous samples. The variation tendency our study gating the role of CD4 To our knowledge, this is the first study investigating the molecular mechanism is critical for an increased understanding of the pathogenesis, early detection, prevention and treatment. It is believed that UC is a consequence of insufficient immune tolerance, and extensive research has focused on regulatory T cells that play a vital role in immunosuppress and immune tolerance mediation [19]. Over the past years, cellular immune function of CD4⁻ T cells receives little attention in the epidemic filed, except for Sartor et al. indicating that CD4⁺ T cells are main immunocytes resulting in inflammatory UC [4]. Currently, the knowledge about the effects of CD4⁻ lymphocyte subsets in the pathopoiesis of human diseases remains limited.

We showed a significantly higher percentage of CD4⁺CD29⁺ T cells in peripheral blood of UC rats, especially those at two weeks after the model were established. Likewise, a significant rise was indicated in peripheral blood of human patients when compared with healthy controls, and a more notable increase was detected in active UC patients relative to the remission patients.

To our knowledge, this is the first study investigating the role of CD4⁺CD29⁺ T cells in colon samples. The variation tendency our study demonstrated is in line with previous CD4⁺CD29⁺ T cell studies focusing on other autoimmune diseases such as multiple sclerosis, and rheumatoid arthritis [20, 21]. It is interesting that a lower percentage of CD4⁺CD29⁺ T cells was found in patients with atopic diseases and mixed connective tissue disease [7, 22]. A plausible explanation for this inconsistency may relate to the different behaviors of CD4⁺CD29⁺ T cells in different diseases, whose pathogenesis may vary extensively.

To expand the understanding of CD4⁺CD29⁺ T cells in UC, we assessed their expression in colon by immunofluorescence. Our results indicated no obvious expression in normal rats and healthy individuals. Interestingly, CD4⁺CD29⁺ T cells were significantly upregulated in UC rats and patients. A relatively smaller rise also took place in other intestinal inflammatory conditions, such as infective enteritis. We therefore inferred that CD4⁺CD29⁺ T cells may be the main effector cell subsets resulting in persistent inflammation for UC. In order to evaluate the effects of CD4⁺CD29⁺ T cells on UC incidence, we evaluated DAI score and MPO, and found a positive association of CD4⁺CD29⁺ T cells with DAI score and MPO. MPO exists in azurophilic granule of neutrophils primarily recruited into the areas of acute inflammations and subsequent UC [23]. Previous groups have reported that MPO is positively associated with severity of intestinal inflammation, and an ideal indicator of UC activity and severity [17, 24, 25]. Consistently, UC patients showed statistical differences in DAI and MPO in our study, with higher levels in active UC patients than in remission patients. Also, we demonstrated a positive correlation of CD4⁺CD29⁺ T cells with DAI score and MPO. Taken together, CD4⁺CD29⁺ T cells might be closely related to the activity and severity of UC.

We additionally investigated the potential mechanisms by evaluating the expression of VCAM-1 via immunohistochemistry. Existing literature has well documented that recruitment of circulating leukocytes, especially the T-effector and T-memory cell to the intestinal mucosa, is a pivotal step in initiation and progression of UC [26]. CD4⁺CD29⁺ T cells have been shown to have greater ability in adhering to vascular endothelium than other CD29 counterparts [27]. Enhanced expression of adhesion molecules possibly lead to CD4⁺CD29⁺ T cell accumulation in inflamed tissues [9]. VCAM-1 as an adhesion molecule is markedly linked with CD29. Collective data have demonstrated that VCAM-1 plays a central role in leukocyte recruitment in colitis and blockade of adhesion molecule and has thera-
peutic effects on DSS colitis [28, 29]. According to Lundberg et al., CD49b/CD29 can regulate neutrophil recruitment and inflammatory responses in dextran sodium sulfate-induced colitis [30]. Therefore, we believe that CD4+CD29+T cells infiltrate to inflamed intestinal tissues most likely through combination with VCAM-1, triggering neutrophil recruitment and inflammatory activity in UC.

Our study showed no obvious expression of VCAM-1 in normal rats and healthy humans, but did indicate different degrees of expression in UC rats and patients. Concordant with CD4+CD29+T cells, VCAM-1 was highly expressed in 2 weeks rat group and the active UC group. Our findings are supported by a recent study conducted by Gulubova et al., who found higher expression levels of VCAM-1 and D4+CD29+T cells in active UC patients when compared to the remission patients, and no expression in healthy controls [31]. Also, Soriano et al. identified low constitutive expression of VCAM-1, but notably increased after induction of colitis [28]. We demonstrated evidence of a prominent rise in the number of CD4+CD29+T cells at two weeks, an observation consistent with an earlier experimental study [32]. It is widely accepted that chronic gut inflammation in UC may arise from an unregulated immune response to components of the normal gut flora [33, 34]. Recruitment of T-effector and T-memory cells that recognize antigens in gut lumens is markedly associated with disease state such as acute intestinal inflammation [26]. CD29 could be detected in cellular membrane of lymphocytes, monocytes, and neutrophils under certain conditions [35]. CD4+CD29+T cells as memory T cells differentiated from CD4+T cells may function significantly in repeated response to antigen.

In summary, we found notably upregulated expression of CD4+CD29+ T cells in peripheral blood and colon of UC rats and patients. Pearson correlation analysis indicated a positive association of CD4+CD29+T cells with disease activity and severity. We also noted CD4+CD29+T cell migration to the inflamed colon tissues via combination with VCAM-1. These results support the hypothesis that CD4+CD29+T cells may act as effector cell subsets leading to persistent inflammation and also blamed for the repeated inflammation attacks of UC. Considering the limited sample, our findings should be treated as preliminary and additional studies are warranted to validate these findings possibly in a larger number.

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Disclosure of conflict of interest

None.

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